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EVALUATION OF THE *IN VITRO* CYTOTOXICITY OF *GANODERMA COLOSSUS* EXTRACTS ON CANCEROUS AND NORMAL CELLS: INSIGHTS INTO THEIR ANTICANCER POTENTIAL

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ABBREVIATIONS

AMSL : Above Mean Seal Level
AO/EB : Acridine Orange/Ethidium Bromide (AO/EB) Staining
Bcl-2 : Bcl-2 (B-cell lymphoma 2), encoded in humans by the BCL 2 gene
BCL-2 : B-Cell Lymphoma 2 protein family
CE : Chloroform Extract
Ct : Cycle Threshold
DAPI : Diamidino-2-Phenylindole
DMEM : Dulbecco's Modified Eagle Medium
DMSO : Dimethyl Sulfoxide
DPPH : Diphenyl-1-Picrylhydrazyl
EE : Ethyl acetate Extract
FACS : Fluorescence-activated Cell Sorting
GAE : Gallic Acid Equivalent
GAPDH : Glyceraldehyde-3-phosphate Dehydrogenase
GC- MS : Gas Chromatography Mass Spectrometry
IFA : Immunofluorescent Assay
IUCN : International Union for Conservation of Nature
LC :Least Concern
ME : Methanolic Extract
MTT : Dimethylthiazol-2,5 diphenyltetrazolium bromide
NC : Negative Control
NTC : Negative Temperature Coefficient
NTC : Non-Template Control
PBS : Phosphate Buffered Saline.
PC : Positive Control
QE : Quercetin Equivalent
qRT-PCR : Real-Time Quantitative Reverse Transcription PCR
ROS : Reactive Oxygen Species
RT : Retention Time
TP^{53} : Tumor Protein 53
TPC : Total Phenolic Content

ABSTRACT

This study evaluated the antioxidant and anticancer potential of medicinal mushrooms *Porodaedalea pini*, *Phellinus* sp., and *Ganoderma colossus* using *in vitro* and *in silico* models. Among various solvent extracts, the methanol extracts exhibited the highest antioxidant activity, with *G. colossus* showing superior performance. Cytotoxicity assays revealed that ethyl acetate extracts of *G. colossus* and *Phellinus* sp. were most effective, with *G. colossus* demonstrating greater selective cytotoxicity against MCF-7 breast cancer cells compared to HeLa cells and sparing normal HEK-293 cells. Mechanistic studies on *G. colossus* indicated P53-mediated apoptosis through cell cycle arrest, DNA damage, and activation of caspase-3, alongside downregulation of the anti-apoptotic factor BCL-2. Purification of the ethyl acetate extract identified a fraction with high cytotoxicity, containing bioactive compounds that strongly interacted with cancer-related proteins like EGFR, HER2, BCL-2, and MDM2 in molecular docking studies. These findings suggest *G. colossus* as a promising source of natural anticancer agents, warranting further research to isolate and develop its bioactive compounds.

1. Introduction and Review of Literature

1.1 Introduction

The exploration of natural sources for medicinal drug discovery has been an enduring focus of scientific research. Historically, natural products and traditional medicine systems have been instrumental in identifying therapeutic agents, especially for cancer and infectious diseases (Atanasov et al., 2021). Compared to synthetic derivatives, natural products offer unique features, posing both advantages and challenges to the drug discovery process (Thomford et al., 2018; Atanasov et al., 2021). Among these, macro-fungi, particularly mushrooms are of significant interest due to their dual roles as nutritional resources and repositories of bioactive compounds with therapeutic potential.

Mushrooms have been consumed as food and used in traditional medicine for centuries, valued for their distinct flavour, nutritional content, and health benefits (Stoffel et al., 2019). They are rich in proteins, carbohydrates, dietary fiber, essential vitamins (e.g., riboflavin, pantothenic acid, ascorbic acid), and minerals such as iron, calcium, and phosphorus (Singh, 2017; Painuli et al., 2020; Lesa et al., 2022). Their low cholesterol content, coupled with a high proportion of unsaturated fatty acids, further enhances their appeal as a healthful dietary choice (Koyyalamudi et al., 2009). Beyond their nutritional attributes, mushrooms are increasingly

recognized for their medicinal properties, including antioxidant, antimicrobial, and anticancer effects (Adeniyi et al., 2018 ; Łysakowska et al., 2023).

The concept of medicinal mushrooms has gained global prominence, driven by their ethnopharmacological relevance and mounting evidence of therapeutic efficacy (Panda et al., 2022a). Their safe use in clinical settings, often in conjunction with conventional therapies such as chemotherapy or radiation, highlights their potential in cancer treatment (Venturella et al., 2021). Despite advancements in medical science, cancer remains the leading cause of death worldwide, underscoring the urgency for novel therapeutic strategies (Siddique & Kiani, 2020).

Natural remedies derived from plants and fungi are a focal point for anticancer drug discovery due to their proven efficacy and lower toxicity. While over 60% of current anticancer drugs are natural product derivatives, no such drugs have yet been derived from mushrooms (Panda et al., 2022b). This is notable given the longstanding claims of mushrooms' anticancer properties, supported by traditional usage and contemporary research in countries like Japan, China, Korea, and the USA (Figueiredo & Régis, 2017). *In vitro* and animal studies suggest that mushrooms possess preventive, corrective, and therapeutic effects against various cancer types (Figueiredo & Régis, 2017; Krishnakumar et al., 2023). The bioactive compounds in mushrooms, such as polysaccharides, phenolics, and terpenoids, are thought to contribute to their anticancer activity by modulating key pathways involved in cell proliferation, apoptosis, and immune responses. Additionally, even non-edible or poisonous mushrooms may harbour bioactive molecules with anticancer potential (Lima et al., 2012; Elkhateeb & Daba, 2022). However, despite numerous studies, many mushroom species remain underexplored for their medicinal potential, presenting an untapped opportunity for discovering novel therapeutic agents (Hawksworth, 2001; Wasser, 2014).

With the increase in burden of cancer and the limitations of existing therapies, the study of mushrooms as complementary and alternative treatments holds significant promise. The present study aimed to evaluate the cytotoxic and therapeutic properties of selected mushroom species, with a focus on their bioactive compounds, mechanisms of action, and potential applications in anticancer drug discovery.

Aim of the Study

To investigate the cytotoxicity, phytochemical composition, and anticancer mechanisms of mushroom extracts, with a focus on *Ganoderma colossus*, through bioactive compound characterization and molecular interaction studies to validate their potential as anticancer agents.

Objectives of the Study

1. To identify and correlate bioactive compounds in mushroom extracts with their biological activities through phytochemical analysis.
2. To evaluate the cytotoxic effects of mushroom extracts on cancerous (MCF-7, HeLa) and normal (HEK-293) cells.
3. To elucidate the anticancer mechanisms of *Ganoderma colossus* extracts by analysing cell cycle arrest, DNA damage, apoptosis, and gene expression modulation in MCF-7 cells.
4. To characterize bioactive molecule through fractional purification and identification of its molecular interactions using GC-MS and docking studies, while validating its anticancer potential

1.2 Review of Literature

1.2.1 Mushrooms of Western Ghats

The Western Ghats of India, spanning 1,600 km across six political states (Gujarat, Maharashtra, Goa, Karnataka, Tamil Nadu, and Kerala), is a diverse mountain range. In Karnataka and Kerala, the Western Ghats boast a rich variety of vegetation, encompassing grasslands, shola forests, deciduous forests, moist-dry deciduous forests, evergreen forests, semi-evergreen forests, and scrub jungles, distributed across different altitudinal ranges (500 to 1,200 AMSL). Karun & Sridhar (2017) compiled information on 51 edible wild mushrooms in the Western Ghats, detailing their habitats, substrates, mutualistic associations, availability, edibility, and preservation methods. Bioactive potential in uncooked and cooked conditions was evaluated for two edible mushrooms, namely *Auricularia auricula-judae* and *Termitomyces umkowaan*, found in the Western Ghats (Karun et al., 2016). Sudheep & Sridhar (2014) reported the nutritional composition of two wild mushrooms consumed by the tribals in the Western Ghats.

Ragupathi et al. (2018) studied the antioxidant activity of some wild mushrooms from the southern Western Ghats of India. Toxicity assessment of wild mushrooms from the Western Ghats was also reported, involving *in vitro* and sub-acute *in vivo* studies (Latha et al., 2018) . The

literature survey indicates a range of studies on mushrooms in the Western Ghats, covering diverse aspects such as diversity, nutritional values, and medicinal properties.

1.2.2 Medicinal Values of Mushrooms

Medicinal mushrooms have a rich history of use in folk medicine. According to Kabir et al. (1988), *Ganoderma lucidium* has been employed in traditional medicine in China and Japan to treat conditions such as hepatopathy, chronic hepatitis, hypertension, arthritis, bronchitis, asthma, and gastric ulcers. Mau et al. (2001) noted that the applications of mushrooms for maintaining health were recorded as early as 100 A.D in China. Traditional uses include lubricating the lungs (*Tremella fuciformis*), tonifying the kidneys (*Cordycepes sinenisis*), and reducing excessive dampness (*Grifola umbellate*) (Chang & Wasser, 2012a). Ramesh & Pattar (2010a) reported that mushrooms produce biologically active compounds, mainly associated with the cell wall, contributing to enhanced immunity and tumor-retarding effects. Mushrooms are incorporated into health tonics, tinctures, teas, soups, and various healthy food dishes (Gopal et al., 2022), as well as herbal formulas (Bains et al., 2021). Functional mushrooms have been shown to modulate the immune system, lower blood pressure, and blood lipid concentration, and inhibit tumors, inflammation, and microbial action (Thakur & Singh, 2013).

1.2.3 Anticancer Activity of Mushrooms

Studies on the anticancer activity of mushrooms involve both *in vitro* and *in vivo* experiments. It has been reported that around two hundred species of mushrooms significantly inhibit the growth of various tumors (Patel & Goyal, 2012). Researchers have isolated active polysaccharides with antitumor activity, identifying them as (1-3)-β-glucopyranans with a (1-6)-β-D-glucosyl branch containing protein (Mizuno, 1999). Ethnopharmaceutical studies on mushrooms, specifically regarding cancer therapy, have been documented (Özmen & Değirmenci, 2021).Valverde et al. (2015) demonstrated that extracts of *Calvatia craniiformis* efficiently inhibited tumor growth, reducing tumor size clinically and increasing the expression of the caspase-8 gene product in tumor tissue. Dose and time-dependent antitumor activity of aqueous and methanolic extracts of *C. craniiformis* has been reported (Jameel et al., 2018). Complex proteins in mushrooms exhibit immune modulating as well as direct cytotoxic activity towards cancer cells (Xu et al., 2011). Mushroom polysaccharides are reported to prevent carcinogenesis, metastasis, and display immune cell-mediated anticancer activity (Zhang et al., 2007a). Polysaccharides with antitumor activity in mushrooms predominantly belong to glucans with β-(1→3) bonds in the main chain and β-(1→3) bond side branches, essential for biological activity. Ren et al. (2012) demonstrated that high-molecular-weight mushroom polysaccharides exhibit more efficient antitumor activity than low-molecular-weight molecules. Isolated

mushroom polysaccharides, such as lentinan, have shown properties that help to treat cancer (Ooi & Liu, 2012).

As specified earlier, cancer stands as a major global cause of death, and the current anticancer therapeutics lack target specificity, leading to severe side effects and complications in clinical management. This underscores the pressing need for novel, effective, and less-toxic therapeutic approaches. In this regard, mushrooms emerge as promising candidates with potential anticancer properties, warranting exploration and characterization through research. The diverse mushroom species found in the Western Ghats offer a unique opportunity to identify new molecules, previously unexplored for their antitumor potential. The present study focuses on exploring anticancer molecules from specific mushroom species in the Western Ghats of coastal Karnataka and Kerala. Following a literature survey, *Ganoderma* and *Phellinus* mushroom species were selected for the study due to their traditional medicinal usages.

1.2.4 *Phellinus* species - a Source of Medicine

Phellinus, is a vast and widely distributed genus belonging to the family Hymenochetaceae within the class Basidiomycetes, is extensively utilized in China, Japan, Korea, and various other Asian countries (Chen et al., 2016). With a traditional Chinese medicine history dating back 2000 years, its medicinal applications primarily involve the treatment of hemorrhage and homeostasis (Azeem et al., 2020). Numerous studies have highlighted the positive therapeutic effects of *Phellinus* species on various diseases (Zhu et al., 2008; Chen et al., 2016; Azeem et al., 2020).

In Kerala, India, *Phellinus* is referred to as "pilamangal" (pila = jackfruit tree; mangal = turmeric), while in Maharashtra, it is known as "phansomba" (phanos = jackfruit; alombe = mushroom) (Vaidya & Lamrood, 2000). *Phellinus* has been a crucial component in various Ayurvedic formulations, particularly used to treat oral cancers through an Ayurvedic decoction called "Kashayam." Additionally, *Phellinus* has been employed to address conditions such as brain tumors, rectal cancer, intestinal cancer, throat cancer, numbness, and excessive salivation, particularly in Karnataka (Sridhar & Deshmukh, 2019). In a recent study, hot water extracts from *Phellinus* mushrooms demonstrated significant potential in terms of anticancer efficacy when compared to other mushrooms (Mizuno, 2000). The historical use and diverse applications of *Phellinus* in traditional medicine emphasize its potential as a valuable source of medicinal compounds.

1.2.5 Medicinal Values of *Ganoderma* Mushroom species

Species belonging to the genus *Ganoderma* boast the longest historical usage for medicinal properties, dating back at least four thousand years (Kumar et al., 2021). It is known as Reishi in Japan and Ling Chu or Ling Zhi (mushroom of immortality) in China and Korea. Ganoderma has a rich history in traditional medicine. It has been widely employed to treat various conditions, including hepatopathy, chronic hepatitis, nephritis, hypertension, insomnia, bronchitis, asthma, and gastric ulcers (Bhardwaj & Misra, 2018; Bulam et al., 2019).

Scientific studies have substantiated the medicinal efficacy of substances extracted from *Ganoderma,* showcasing their ability to reduce blood pressure, lower cholesterol levels, and inhibit platelet aggregation (Hunt & Vorburger, 2002). The isolated terpenes and polysaccharides from *Ganoderma lucidium* have demonstrated anticancer activity, and their use in combination with other known anticancer agents has been shown to enhance drug efficacy (Yuen & Gohel, 2005). The extensive historical use and scientifically validated therapeutic properties underscore the significance of *Ganoderma* as a valuable source of medicine.

2. Phytochemical Profiling and Correlation of Bioactive Compounds in Medicinal Mushrooms

Despite remarkable progress in cancer treatment, there is a growing interest in developing novel anticancer agents due to the resistance of cancer cells to existing medications (Hoskin & Ramamoorthy, 2008; Dallavalle et al., 2020). Medicinal mushrooms contain biologically active substances such as polysaccharides, proteins, protein-polysaccharide complexes, steroids, terpenoids, phenols, flavonoids, and dietary fiber, among others, exhibiting anticancer properties (Zong et al., 2012). Polysaccharides isolated from mushrooms have demonstrated anti-carcinogenic and anti-metastasis properties, along with immune cell-mediated anticancer activity (Zhang et al., 2007b).

The present study was aimed to identify bioactive compounds present in selected mushroom samples, assess their antioxidant activity, determine the cytotoxic potential, and investigate the anticancer mechanism of the highly cytotoxic mushroom samples collected from Western Ghats.

2.1 Collection, Identification and Biochemical Analysis

2.1.1 Introduction

Ganoderma is a potential mushroom species that contains a significant array of bioactive molecules (Parepalli et al., 2021). Traditionally, this species was used to treat various diseases like AIDS and cancer (Cör et al., 2018). The antioxidant properties of *Ganoderma* help to alleviate the oxidative damage, which in turn aids in reducing mutation, carcinogenesis, and cancer development (Sheena et al., 2003). The anticancer and anti-metastatic properties of various products derived from *Ganoderma* have been demonstrated in both *in vivo* and *in vitro* studies (Kladar et al., 2016). *Ganoderma colossus* is a unique *Ganoderma* species have different morphological features compare to majority of other *Ganoderma* species. Despite its potential as a medicinal mushroom, it has not been explored extensively. Currently there are only limited number of studies addressing the anticancer potential of the mushroom *Ganoderma colossus.*

The *Phellinus* species are rich in bioactive molecule. The existence of terpenoids, phenolic chemicals, and polysaccharides has been determined through research studies on microchemical analysis. These compounds show many types of biological activities, including anticancer, antioxidant, anti-angiogenic activities (Sułkowska-Ziaja et al., 2021). Researchers have recognized Phellinus species as a promising functional food due to their diverse biological activities. In earlier times, *Phellinus* sp. was traditionally utilized in eastern countries to alleviate diarrhea, hemorrhage, and gastrointestinal dysfunction conditions. (He et al., 2021). There are about 220 species of *Phellinus*, and most of these mushroom species remain untapped. This indicating the significance of the study of *Phellinus* mushroom.

Numerous species of edible and non-edible mushrooms have been identified as composed of a diverse array of molecules with high nutritional and medicinal properties. However, the values of these properties vary widely among mushroom species due to the diverse compounds with different solubility in the solvents used for extraction (Elbatrawy et al., 2015a). Soluble polar substances in crude mushroom extracts are believed to exhibit potential antioxidant and anticancer properties. Aqueous extracts of *Lentinula edodes* and *Pleurotus sajor-caju* have demonstrated inhibitory activity against human tumor cell lines, such as laryngeal carcinoma (Hep-2) and cervical adenocarcinoma (HeLa) (Finimundy et al., 2013).

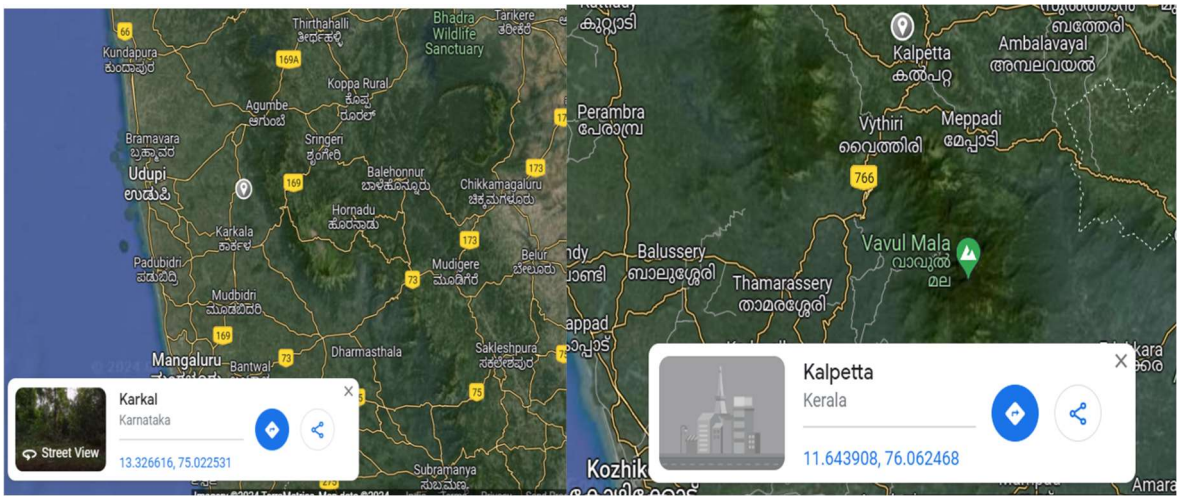
2.1.2 Materials and Methods

2.1.2.1 The Sampling Sites

The mushroom samples were collected from the foothills of the Western Ghats in southern Karnataka and Wayanad, covering Dakshina Kannada and Udupi districts of Karnataka, as well as the Kalpatta region of Wayanad in Kerala.

2.1.2.2 The Collection of Mushroom Samples

The collection of the selected mushroom species was conducted during the monsoon in the years 2018-2019. Initially, ethno-mycological information was gathered from local and tribal communities through direct observations and interviews. A total of three different mushroom samples were collected based on this information.



Google Image of the Sampling Site; Source- https://images.google.com/

To preserve their freshness, the collected samples were carefully packed in wax paper and placed in a cardboard box for transportation to the laboratory. Subsequently, the samples underwent a drying process at 40-50°C in a hot air oven, followed by pulverization in a blender. The powdered samples were then stored for future extraction. Dried specimens were also preserved as a herbarium collection, packaged in paper packets, and stored in the Department of Biosciences, Mangalore University each assigned a unique voucher number for reference. The accuracy of the gathered information was validated through a comprehensive literature survey. For the identification of the collected mushrooms, authoritative books (Dhaheri et al. 2013; Ramesh & Pattar 2010; Chang & Wasser 2012b; Wang 2012) were referred. Species-level

identification followed standard taxonomic methodologies (David & Spoerke, 2019) and the identification of the mushroom samples were authenticated by experienced taxonomists.

2.1.2.3 Preparation of the Crude Extracts of Mushrooms

The mushroom samples underwent extraction using the standard extraction method, as described by Oshadie et al. (2017). Three different solvents, namely chloroform, methanol, and ethyl acetate, were used for the extraction process. Ten grams of dry powder from each mushroom sample were individually extracted with the three different solvents such as methanol, ethyl acetate and chloroform.

2.1.2.4 Determination of the Yield of the Crude Extracts

The percentage yield of each solvent extract was calculated using following formula:

% Yield = W1/W2 X 100

Where, W1= Weight of the dried extract obtained in grams

W2= Weight of the mushroom powder used for extraction in grams

2.1.2.5 Qualitative Analysis of the Mushroom Sample

A qualitative analysis of the crude extracts of mushroom sample was performed to screen their biochemical constituents such as alkaloids, flavonoids, phenols, carbohydrates, proteins and saponins by following standard protocols (Parekh & Chanda, 2007).

2.1.2.6 Determination of Carbohydrate Content

The total carbohydrate content of the samples was estimated using the Anthrone method (Ludwig & Goldberg, 1956) with some modifications.

2.1.2.7 Determination of Total Protein Content

The protein content of the extracts was determined according to Lowry's method (Lowry et al., 1951) with some modifications.

2.1.2.8 Statistical Analysis

All experiments were conducted in triplicate, and the data were analysed and expressed as mean ± standard deviation (SD). Statistical analysis involved Analysis of Variance (2-way ANOVA), followed by Tukey's multiple comparison tests using Graph pad Prism 8. The p-values were calculated and indicated by '*'.

2.1.3 Results

2.1.3.1 Identification of the Mushroom

Mushroom samples were collected from the Udupi and Wayanad districts, located in the foothills of the Western Ghats. The collection was based on ethnomycological information obtained from local and tribal communities, supplemented by direct observation. Taxonomic studies and identification were validated by a taxonomist, confirming that the collected mushrooms were *Ganoderma colossus*, *Phellinus pini* (*Porodaedalea pini*) and *Phellinus* sp.

2.1.3.2 Extraction Yield

The results (Fig. 1) of the study revealed a polarity-dependent increase in extraction efficiency. Among the three mushrooms investigated, the methanol extract of *G. colossus* exhibited the highest yield (17.46±2.65%), followed by *P. pini* (9.64±1.19%) and *Phellinus* sp. (4.58±0.77%). This variation in the yield indicated that the extraction efficiency of crude extracts differed among species. The findings affirmed the presence of a greater number of polar compounds in the *G. colossus* and *P. pini* compared to *Phellinus* sp. Additionally, methanol emerged as the most effective solvent in terms of high yield for extracting compounds when compared to ethyl acetate and chloroform.

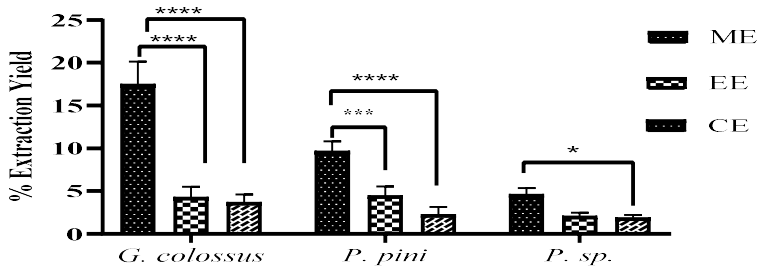


Fig (1): The Percentage Extraction Yield of the Samples

The results represent mean ±SD. Where ME, methanol extract; EE, ethyl acetate extract; CE, chloroform extract; $^{*}p< 0.05$, $^{***}p < 0.001$ and $^{****}p < 0.0001$

2.1.3.3 Qualitative Analysis of the Mushroom Samples

Qualitative analysis was conducted to screen the macro-fungal biochemical constituents of the selected mushrooms, and the results are presented in table 1. The phytochemical screening indicated that the selected mushrooms contain a variety of bioactive compounds, including phenolics, flavonoids, terpenoids, tannins, saponins, alkaloids, carbohydrates, steroids, and proteins.

Table (1): Qualitative Analysis of the Samples

Chemical Constituents	**Tests**	**Mushroom Samples**		
		G. colossus	***P. pini***	***Phellinus* sp.**
Phenol	Ferric chloride	+	+	+
	Lead acetate	+	+	+
Flavonoids	Lead acetate	+	+	+
	Alkaline reagent	+	+	+
Terpenoids	Salkowski test	+	+	+
	Chloroform and H_2SO_4.	+	+	+
Tannins	Ferric chloride	+	+	+
	Lead acetate test	+	+	+
Saponins	Form test	+	+	+
	Froth test	+	+	+
Alkaloids	Mayer's test	+	+	+
	Hager's test	+	+	+
Carbohydrates	Molisch test	+	+	+
	Benedict's test	+	+	+
Steroids	Salkowski test	+	+	-
	Liberman Burchard test	+	+	-
Proteins	Ninhydrin test	+	+	+
	Biuret test	+	+	+

Legend:(-) absent; (+) present. ME, methanolic extract; EE, ethyl acetate extract; CE, chloroform extract.

2.1.3.4 Carbohydrate Content

The carbohydrate content was found to be high in the methanol extract of all the selected mushrooms. Specifically, *P. pini* exhibited a higher carbohydrate content (71.8±2.18mg/100g, $p<0.0001$) compared to the other two mushrooms. The methanol extracts of *G. colossus* exhibited a carbohydrate content of 32.08±2.5 mg/100g, while the ethyl acetate extract demonstrated the

lowest carbohydrate content at 9.98±3.0 mg/100g. In the case of *Phellinus* sp., the methanol extract revealed a carbohydrate content of 12.1±3.7 mg/100g, with the ethyl acetate extract showing a lower carbohydrate content compared to the chloroform extract. However, in the case of *P. pini*, there was a polarity-dependent increase in solubility, resulting in higher carbohydrate content.

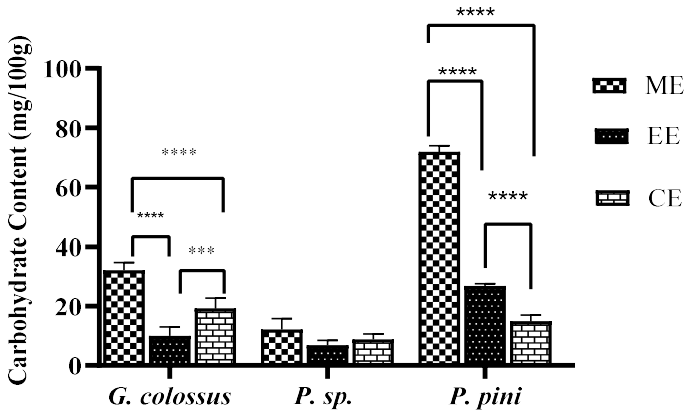


Figure (2): Comparison of the Carbohydrate Content

The result expressed in Mean ± SD**.** Where ME; methanol extract; EE, ethyl acetate extract; CE, chloroform extract; ***p< 0.001 and ****p< 0.0001.

2.1.3.5 Protein Content

Among the three selected mushroom species, the methanol extract of *P. pini* exhibited the highest protein content (226.2±3.6 mg/100g; p<0.0001) compared to *Phellinus* sp. (139.08±5.8 mg/100g) and *G. colossus* (13.9±1.5 mg/100g). The ethyl acetate extract of *Phellinus* sp. (177.7±6.8 mg/100g, p<0.0001) revealed the second-highest protein content. Interestingly, the ethyl acetate extracts of *G. colossu*s and *Phellinus* sp. displayed higher protein content (50.6±3.9 mg/100g, p<0.0001 and 177.7±6.8 mg/100g, p<0.0001, respectively) compared to the methanolic extract. The chloroform extract showed the least amount of protein among the three extracts.

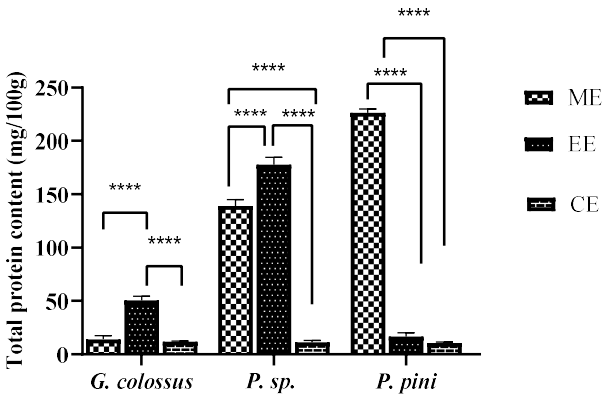


Figure (3): Comparison of the Protein Content

The result expressed in Mean ± SD. Where ME; methanol extract; EE, ethyl acetate extract; CE, chloroform extract; $^{****}p< 0.0001$

2.1.4 Discussion

The identified mushroom samples, *Ganoderma colossus*, *Porodaedalea pini* (*Phellinus pini*) and *Phellinus* sp., were found to contain a diverse array of bioactive compounds. The solubility of these compounds varied across different mushroom extracts, indicating differences in their bioactive content. Existing literature suggests that the biological activity and extraction yield of phytochemicals tend to increase with the polarity of the solvent and the chemical characteristics of the extraction solvent (Fang et al., 2014). This is in agreement with the findings of (Nawaz et al., 2020), which reported that polar solvents shown the highest extraction yield.

Qualitative analysis of the mushroom samples revealed the presence of phenolics, tannins, saponins, alkaloids, terpenoids, carbohydrates, and proteins. These secondary metabolites are known for their antioxidant activity (Walia let al., 2022). The high carbohydrate content in the *Phellinus pini* extracts emphasizes the report of the presence of different polysaccharides in the *Phellinus* species (Kou et al., 2024). Additionally, the polysaccharides present in mushrooms are attributed to a range of medicinal properties (Wasser, 2002; Martyasari et al., 2021).

Among the solvent extracts, the methanolic extract of *P. pini* and the ethyl acetate extract of *Phellinus* sp. demonstrated the highest protein content compared to the other solvents. The study conducted by Kim et al. (2006) reported the presence of protein polysaccharide complexes in the *Phellinus* species. Conversely, the chloroform extracts exhibited the lowest protein content. The mushroom *G. colossus* exhibited less protein content compared to other two mushroom

samples, The variation in protein content may be attributed to differences in the number and nature of amino acids present in the proteins. Certainly, the regulation of gene expression, involving both down- and up-regulation, is a critical factor influencing the total protein content within an organism. This intricate process is shaped by species-specific characteristics and responds dynamically to the specific requirements of the organism (Wu et al., 2021). Meera (2022) reported the polysaccharide protein and protein derived from various medicinal mushrooms, used to enhance immune system and improved disease resistance.

This study highlights the importance of selecting an appropriate solvent for extraction, with polar solvents showing superior extraction yield. The observed higher yield and solubility in polar solvents suggest the presence of more polar-soluble components and hydrophilic substances in the mushroom samples. This information emphasizes the necessity of considering solvent polarity for optimal extraction of bioactive compounds in future studies.

2.2 Assessment of Antioxidant Activity

2.2.1 Introduction

Reactive oxygen species (ROS), prevalent free radicals in living organisms, can damage crucial biomolecules. While ROS generation is inevitable due to aerobic metabolism, their harmful effects are mitigated by antioxidants. Mushrooms are known to contain numerous antioxidant molecules capable of neutralizing free radicals (Ferreira et al., 2009; Sánchez, 2016).

Given the role of free radicals in health disorders, exploring molecules with free radical scavenging activity is crucial. Mushrooms, as valuable antioxidant sources, have garnered significant research interest for their potential in preventing diseases, including neurodegenerative, cardiovascular, and cancer-related conditions (Manach et al., 2004; Calabrese et al., 2010).

This study was focussed to examine how solvent polarity affects the extraction of secondary metabolites and antioxidant activity, highlighting the relationship between solvent choice and the biochemical potential of mushrooms.

2.2.2 Materials and Methods

2.2.2.1 Determination of Total Phenolic Content (TPC)

The total phenolic content of the extracts from the mushroom was assessed using the Folin Ciocalteu method (Slinkard & Singleton, 1977). Absorbance was measured at 720 nm, and

a calibration curve was constructed using standard gallic acid at varying concentrations (5 µg/ml to 360 µg/ml).

2.2.2.2 Determination of Total Flavonoid Content (TFC)

The TFC of the extracts was determined through the aluminium chloride method (Zhishen et al., 1999). Quercetin was employed as a standard, and the absorbance was measured at 510 nm using a UV Spectrophotometer to generate a calibration curve.

2.2.2.3 Determination of DPPH Radical Scavenging Activity

The radical scavenging activity of the samples was determined using the DPPH methanol assay (Brand-Williams et al., 1995) with some modifications, and absorbance was measured at 518 nm.

$$\% \text{ Inhibition} = [(A_{Control} - A_{sample}) / A_{control}] \times 100$$

2.2.2.4 Statistical Analysis

The statistical analysis was conducted as outlined on page 10. The statistical corelation between total phenolic content, total flavonoid content and antioxidant activity for all the samples done by Pearson correlation using GraphPad Prism 8.

2.2.3 Results

2.2.3.1 Total Phenolic Content (TPC)

The methanolic extract exhibited a high phenolic content for all the mushrooms studied. Among the mushrooms, *P. pini* (Methanol extract) displayed the highest TPC (158.5±3.8 mg GAE/g) and the ethyl acetate extract of this mushroom shown 126.7±3.6 mg GAE/g. The *Phellinus* sp. (88.2±5.3 mg GAE/g) and *G. colossus* (58.0±3.2 mg GAE/g) showed total phenolic content. The phenolic content of all the mushrooms increased with rising solvent polarity.

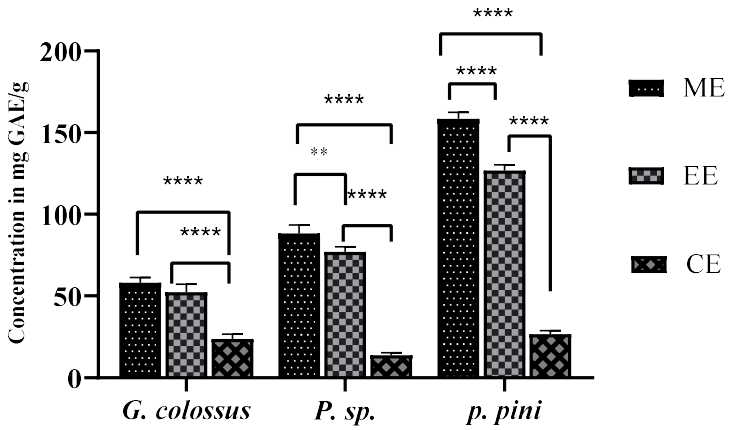


Figure (4): Comparison of Total Phenolic Content

Where ME; methanol extract; EE, ethyl acetate extract; CE chloroform extract

**p <0.01 and ****p< 0.0001(comparison between the extracts)

2.2.3.2 Total Flavonoid Content

The mushroom *P. pini* (ME) exhibited high flavonoids solubility. Both *P. pini* and *Phellinus* sp. demonstrated a polarity-dependent increase in flavonoid content. However, the ethyl acetate extract of *G. colossus* exhibited significantly higher activity compared to the other two extracts ($p < 0.0001$).

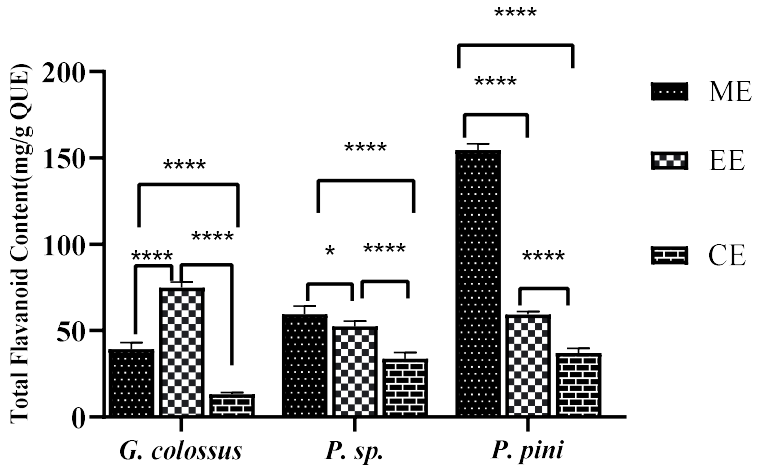


Figure (5): Comparison of Total Flavonoid Content

The results are presented as Mean ± SD, where ME represents methanol extract, EE denotes ethyl acetate extract, and CE signifies chloroform extract. Statistical significance is indicated as follows *$p < 0.05$ and ****$p < 0.0001$(comparison between the extracts).

2.2.3.3 Antioxidant Activity

Among the three mushrooms used in the study, the methanol extract of *G. colossus* (75.1±4.9%) and *P. pini* (71.4±3.6%), demonstrated significant radical scavenging activity compared to the other extracts, while the ethyl acetate extract of the *Phellinus* sp. (71.4±3.6%) shown comparatively higher activity than other extracts as illustrated in Fig. (6).

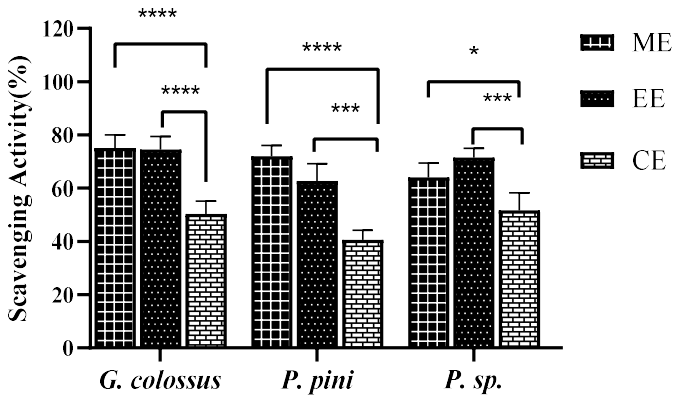


Figure (6): Comparison of DPPH Activity

The results are presented as Mean ± SD. Where ME - methanol extract; EE - ethyl acetate extract; CE - chloroform extract. Statistical significance is denoted as follows:

*p < 0.05, ***p < 0.001 and ****p <0.0001

2.2.3.4 Correlation between TPC and TFC and Antioxidant Activity

The corelation between total phenolic content, total flavonoid content, antioxidant activity was done for all the samples. The *P. pini* phenolics and DPPH showed a strong positive corelation (R^2= 0.995). There was a non-significant correlation observed between phenolics, flavonoids and antioxidant activity of the other two samples.

2.2.4 Discussion

Natural antioxidant molecules are in high demand due to the adverse effects of synthetic alternatives (Engin et al., 2011). Phenolic and flavonoid compounds, known for neutralizing free radicals, are vital natural antioxidants (Tumilaar et al., 2024). In this study, methanol extracts from all three mushrooms exhibited high phenolic content, consistent with the efficacy of polar solvents in extracting polyphenols (Galanakis et al., 2013).

The mushroom *Phellinus pini* showed significantly higher phenolic and flavonoid content, aligning with reports that certain species, including *P. pini*, produce flavonoids (Pukalski & Latowski, 2022). Flavonoid content varied, with the ethyl acetate extract of *Ganoderma colossus* containing the highest levels, corroborating findings by Gautam et al. (2022). Similarly, the methanol extract of *P. pini* and *P*. sp. displayed the highest flavonoid content among their respective extracts, aligning with Tel et al. (2015a).

Antioxidant potential was highest in the methanol extracts of *G. colossus* and *P. pini*, with the ethyl acetate extract of *G. colossus* showing comparable activity. However, phenolic and flavonoid content showed a non-significant correlation with antioxidant activity of *G. colossus* and *P.* sp.

Thus, methanol is a preferred solvent for extracting high yields of bioactive compounds, including phenolics and flavonoids, and for achieving strong antioxidant activity. Ethyl acetate, as a secondary option, demonstrated efficacy in extracting bioactive molecules. However, the potential oxidative stress and cytotoxicity induced by more polar components warrant further investigation (Srivastava et al., 2021).

3. Cytotoxic Evaluation of Medicinal Mushroom Extracts on Cancerous and Normal Cells

3.1 Introduction

Cytotoxicity serves as a preliminary study aimed at determining the potential anticancer activity of test samples. This analysis helps in understanding the impact of the samples on cell growth, reproduction, and morphological alterations (Tel et al., 2015b). Cell proliferation is a crucial indicator for elucidating the pathways involved in cell survival or death following exposure to various substances (Adan et al., 2016). Previous research has indicated that crude extracts from solvents with nonpolar or intermediate polarity exhibit more potent cytotoxicity against cancer cells than those with high polarity (Tel et al., 2015b). Moreover, extracts from different mushrooms using the same solvent may possess varying degrees of cytotoxicity (Elbatrawy et al., 2015b).

The effects of a substance on cells can be either cytotoxic, causing cell death, or cytostatic, preventing cell division. Cell cytotoxicity and proliferation assays are essential for estimating how cells respond to drugs or other chemical agents. These assays are frequently employed in pharmaceutical investigations to understand how a drug affects cells. The ability of

a compound to kill cancer cells is typically quantified by the IC50 value, which represents the concentration at which the drug can kill half of the cancer cells (Vine et al., 2009).

3.2 Materials and Methods

3.2.1 Cytotoxicity Analysis

The cytotoxic potential of different extracts from *G. colossus*, *P.* sp., and the standard drug cisplatin was assessed using the MTT assay (Mosmann, 1983). Cancer cells (MCF7 and HELA) and the control line HEK-293 cells were used in the study. Percentage cell viability was calculated using the formula,

Percentage cell viability = [(A - B)/A] *100.

Where,

A = Absorbance of blank

B = Absorbance of sample extract

3.2.2 Morphological Analysis

The morphological changes of cancerous cells after treatment with different concentrations of each extract were observed under a compound microscope. The treated cells were compared with control cells, and images were captured using Zen software.

3.2.3 Statistical Analysis

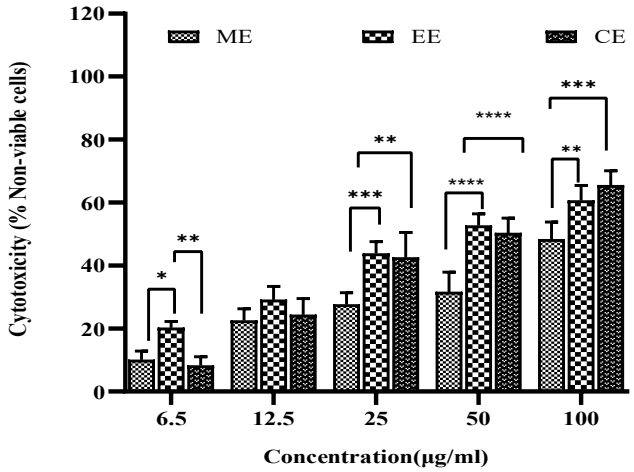
The methodology for statistical analysis is detailed on page 10.

3.3 Results

3.3.1 Cytotoxicity Analysis

Overall, all mushroom extracts demonstrated a dose-dependent increase in cytotoxicity. The ethyl acetate extract from the *G. colossus* mushroom exhibited the highest cytotoxicity. The IC50 values for the extracts in HEK-293, HeLa, and MCF-7 cell lines are provided in Table 3. The *P. pini* extracts, regardless of the solvent, showed lower IC50 values in the normal cell line HEK-293, indicating that it cannot be a target-based product. Consequently, further study on this sample was not pursued.

A)



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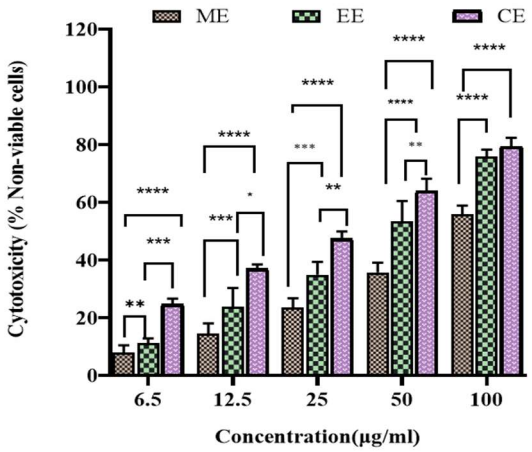

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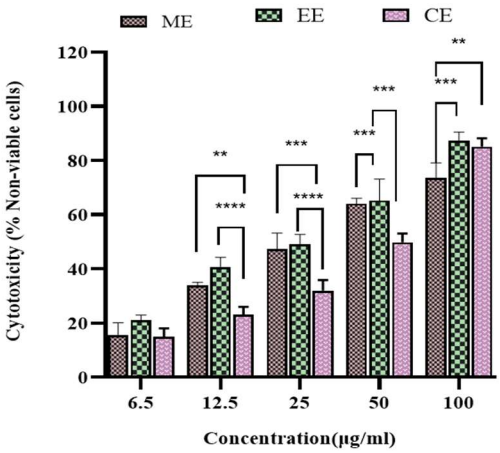
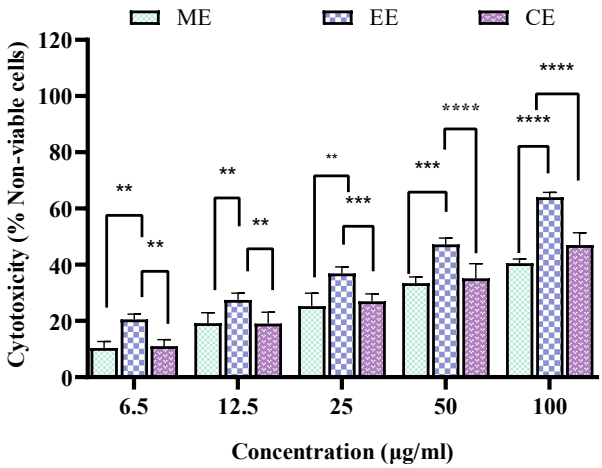


Figure (7): The Cytotoxicity Analysis of the *Phellinus* sp. Extracts in Different Cell Lines

A) HEK-293 B) HeLa and C) MCF- 7; Where EE, ethyl acetate extract, ME, methanol extract and CE, chloroform extract.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$and $^{****}p< 0.0001$, (Comparison between the extracts).

A)



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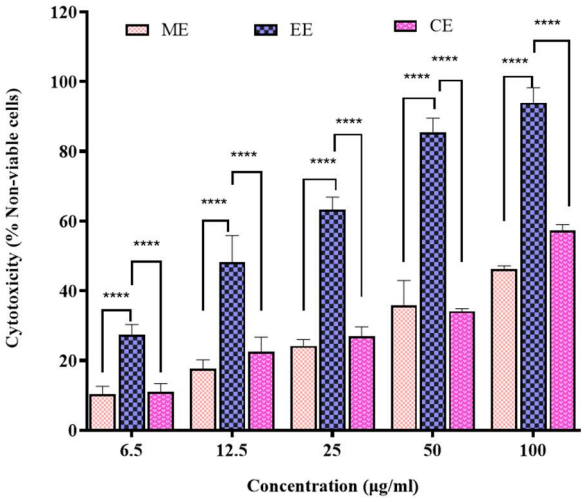

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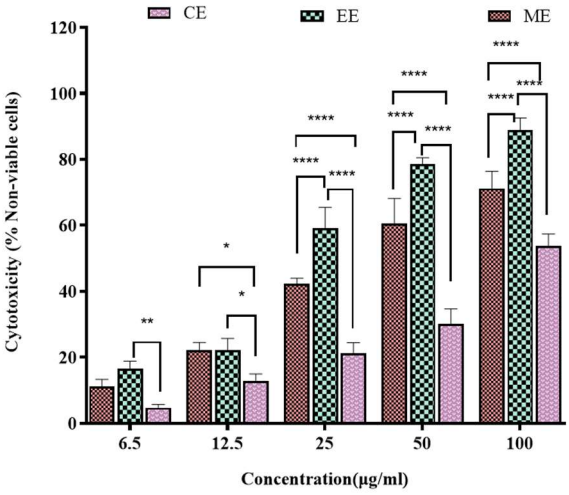


Figure (8): The Cytotoxicity Analysis of the Extracts *G. colossus* in Different Cell Lines

A) HEK-293 B) MCF-7and C) HeLa; Where ME, methanol extract; EE, ethyl acetate extract; CE, chloroform extract; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$(comparison between the extracts).

Table (2): The IC50 Value of the Cytotoxicity Analysis of the Sample Extracts

Cell Lines	*G. colossus*			*Phellinus. sp.*			*P. pini*		
	ME	**EE**	**CE**	**ME**	**EE**	**CE**	**ME**	**EE**	**CE**
HEK-293	123.0±3	65.1±2	106.8±2	99.6±4	60.9±5	61.1±3	54.8±4	36.7±3	30.6±1
HeLa	53.1±6	34.6±1	89.5 ±6	86.9±6	54.3±5	38.1±3	-	-	-
MCF-7	101.9±4	17.2±2	82.9±3	46.2±3	34.4±3	49.5±2	-	-	-

3.3.2 Morphological Changes

The cancer cell lines MCF-7 and HeLa exhibited significant changes in morphology and other features, including detachment from the substratum of the flask, cell membrane blebbing, and irregular nuclei. These alterations were not observed in the negative control (NC) flasks treated with DMSO.

3.4 Discussion

The cytotoxicity analysis of *Ganoderma colossus* extracts on MCF-7 cells revealed that semi-polar and non-polar solvents exhibited higher cytotoxicity compared to polar solvents. These findings align with Elbatrawy et al. (2015c), which highlighted the enhanced cytotoxic effects of semi-polar and non-polar extracts. Similar observations in prior cytotoxicity studies on *Ganoderma* species (Veljović et al., 2017) further corroborate the significant cytotoxicity of these extracts in breast cancer cell lines.

In HeLa cells, the ethyl acetate extract, a semi-polar solvent, demonstrated the highest cytotoxicity, followed by the polar methanol extract, while the non-polar chloroform extract exhibited the least cytotoxicity. The diversity of bioactive compounds with varying solubilities in different solvents highlights the potential presence of anticancer substances across a polarity spectrum. This is consistent with findings by (Suleiman et al., 2022a), who reported significant cytotoxic potential of *Ganoderma lucidum* against colorectal cancer cell lines.

Notably, the IC50 values indicated that the extracts were selectively cytotoxic to cancer cells (MCF-7 and HeLa) while sparing normal cells (HEK-293), suggesting a favorable therapeutic index. However, *Phellinus pini* displayed high toxicity in the normal HEK-293 cell line, prompting its exclusion from further studies. Observed changes in cell viability and morphological features of treated cancer cells underscored the cytotoxic impact of the extracts.

Among the tested extracts, the ethyl acetate extract of *G. colossus* exhibited the most potent activity against MCF-7 cells, positioning it as a promising candidate for further investigation to elucidate its underlying anticancer mechanism

4. Mechanistic Insights into the Anticancer Effects of *Ganoderma colossus* Extracts

4.1 Introduction

The equilibrium between cell proliferation and apoptosis plays a crucial role in normal growth and development (Voss & Strasser, 2020). Convincing evidence suggests that cancer growth is characterized by uncontrolled proliferation and reduced apoptosis. The delicate balance between proliferation and apoptosis is pivotal in determining whether the tumor will expand or regress overall (Tamm et al., 2001; Chen et al., 2016b). Inhibiting cell proliferation and inducing cell death are key processes for controlling tumour growth. The BCL-2 family of proteins regulates programmed cell death and reducing Bcl-2 expression may reactivate apoptosis in cancer cells, enhancing sensitivity to chemotherapeutic drugs (Bathaie et al., 2007; Park et al., 2020).

YH2AX serves as a potent marker for quantifying DNA double-stranded breaks. Phosphorylation of Histone H2AX at serine 139 identifies double-stranded breaks in DNA, facilitating the recruitment of DNA repair proteins to the damaged site. Accumulated YH2AX manifests as a focus on DNA double-stranded breaks in the nucleus (Perri et al., 2015; Akcora-Yildiz et al., 2023). TP53, a marker of cellular stress, is one of the major genes that regulate cell growth, division, and transformation (Marcus et al., 2018a).

4.2 Materials and Methods

4.2.1 AO/EB Staining

Ethidium bromide and acridine orange staining techniques Tyler et al. (1995) were applied to investigate the possible induction of apoptosis by the crude extracts of *G. colossus* in the cancerous cells. Images were acquired using an EVOS-M5000 microscope.

4.2.2 Immunofluorescent Assay (IFA)

The immunofluorescence assay was conducted to examine H2AX foci formation in cancer cells and cells treated with mushroom extracts to assess their damaging activity. The

standard protocol was followed, and coverslip-containing cells were mounted on glass slides with 10 µl of PD-DAPI. Imaging was performed using an EVOS-M5000 microscope.

4.2.3 Flow Cytometry

Cells (0.5M cells/ml) were treated with different extracts of *G. colossus* for 48 hours and processed for flow cytometry following the protocol by (Pozarowski & Darzynkiewicz, 2004). Percentages of cells in different phases of the cell cycle were determined using Incyte software version 7.

4.2.4 RNA Isolation and qRT-PCR for mRNA Detection

RNA from mammalian cells was isolated using triazole reagents per the manufacturer's protocol (Catalog number- T9424, Sigma). The expression of antiapoptotic and checkpoint genes, BCL-2 and TP53, was analyzed using specific primer sequences. GAPDH served as a housekeeping gene, and ΔΔCt values were calculated using Ct values of the housekeeping gene and non-template controls (NTCs).

4.2.5 Immunophenotyping

An immunophenotyping assay with the caspase-3 apoptotic marker was employed to examine its expression, following the protocol by Katheeja et al. (2023). Cells were washed with FACS staining buffer and analyzed with a guava easycyte flow cytometer.

4.2.6 Statistical Analysis

The statistical analysis was performed as described on page 10.

4.3 Results

4.3.1 AO/EB Staining

In this study, the IC-50 value of the extracts induced apoptosis in the cancer cell line. Fluorescent microscopic imaging of ethyl acetate extract-treated MCF-7 cells revealed the highest percentage of apoptotic cells (45.5±2.0%), followed by chloroform extract (39.0±3.0%) and methanol extract (36±2.5%). The apoptosis induced by the positive control, Cisplatin, was 80.0±11.9%. (Fig. 9). The percentage apoptosis induced by the extract compared with positive control. The extract showed significant deviation ($p < 0.0002$), methanol ($p < 0.0001$) and chloroform ($p < 0.0001$) extracts compared to positive control. When compared to untreated cells, extract treated cells showed the increase in the number of apoptotic cells.

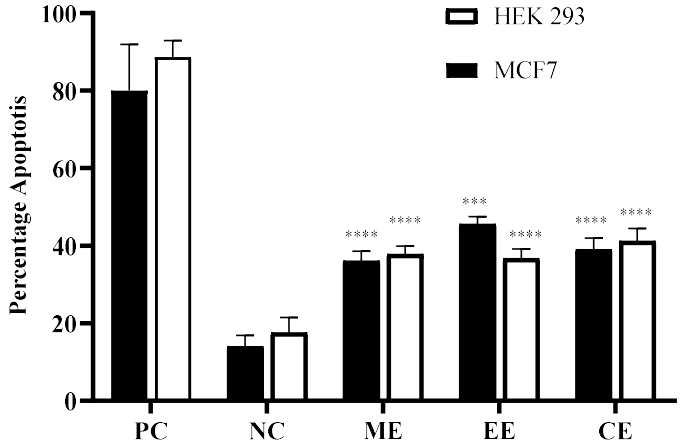


Figure (9): The Percentage of Apoptosis Induced by Various Extracts

Where, PC as the positive control (Cisplatin), NC as the negative control (DMSO), EE as the ethyl acetate extract, ME as the methanol extract, and CE as the chloroform extract.

$^{***}p < 0.001$and $^{****}p < 0.0001$(comparison between PC and the extracts)

4.3.2 Immunofluorescent Assay (IFA)

The immunofluorescent assay (IFA) results are presented in Figure 10, depicting the percentage of flocci accumulation induced by the extracts on HEK-293 and MCF-7 cell lines. The percentage of ϒH2AX accumulation is highlighted for the ethyl acetate extract (36.4±9.2%) and methanol extract (35.1±6.5%), and these extract treatments did not exhibit any significant deviation when compared to the positive control (PC) (40.9±3.4%) in the MCF-7 cell lines.

In HEK-293 cell lines, compared to cisplatin (51.4±6.1%) extracts of *G. colossus* showed a significant deviation. The ethyl acetate extract showed highest percentage of flocci accumulation (36.2±3.1%, $p<0.0167$), followed by the methanol extract (32.2±0.9%, $p< 0.0022$) and least by chloroform extract (CE) (22.3±2.5%, $p<0.0017$).

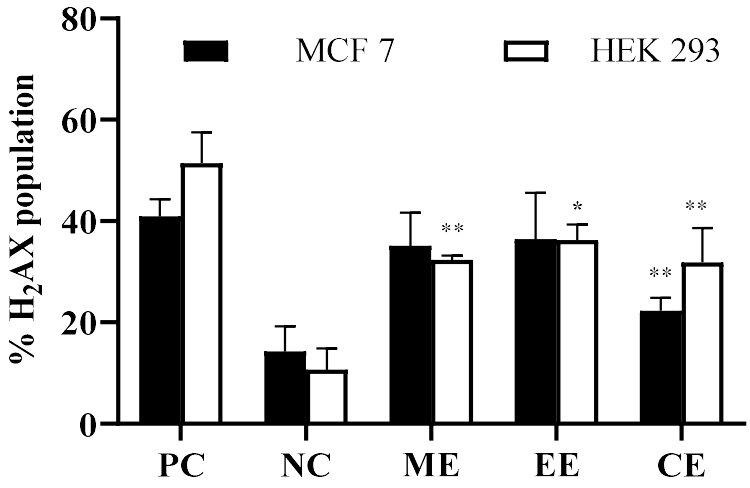


Figure (10): Analysis of the γH2AX Foci Formed by the Treatment with Extracts

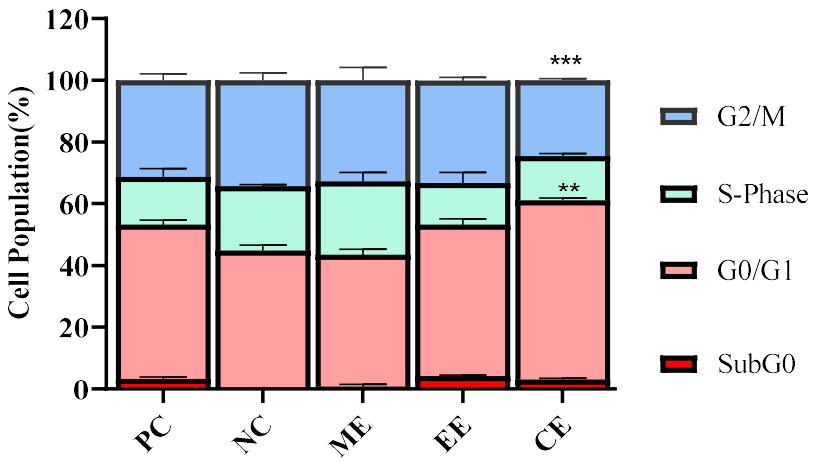
Where PC, positive control (Cisplatin); NC, negative control (DMSO); EE, ethyl acetate extract; ME, methanol and CE, chloroform extract

*p<0.05 and **p<0.01 (Comparison between PC and the extracts)

4.3.3 Flow Cytometry

The flow cytometric analysis was performed to observe the development of changes in the cell cycle phenotype in the MCF-7 cell line, as depicted in Figure 11B. The results showed that ethyl acetate-treated MCF-7 cells exhibit the highest cell cycle arrest at the G0/G1 phase (52.033±3.7%; p<0.0053), followed by the methanol extract (43.3±4.8), in comparison to the negative control (NC) at 42.28±2.19% (Fig. 11B). This arrest was similar to the positive control, cisplatin (50.4±0.7%) (51.23±2.39%, p<0.021), and least observed in the chloroform extract.

A)



B)

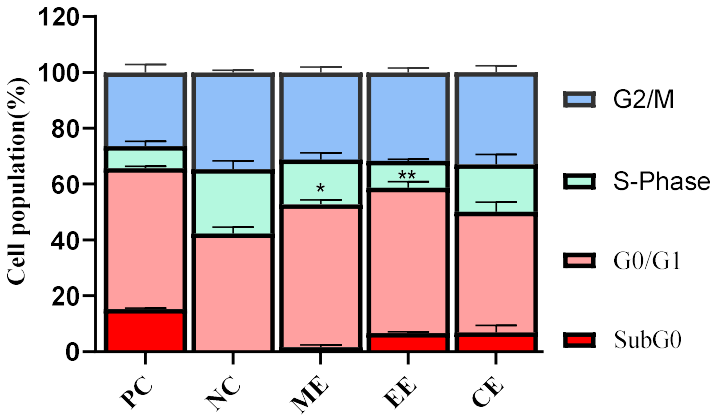


Figure (11): The Percentage Cell Cycle Distribution Induced by the Extracts

A) HEK-293 and B) MCF-7. The result expressed in Mean ±SD. Where PC, positive control (Cisplatin); NC, negative control (DMSO); EE, ethyl acetate extract; ME, methanol extract and CE, chloroform extract

*p<0.05 and **p<0.01 (Comparison between the NC and the extracts).

4.3.4 RNA Isolation and qRT-PCR for mRNA Detection of TP53 and Bcl-2

In the process of RNA isolation and qRT-PCR for detection of mRNA associated with TP53, the expression analysis revealed that the treatment with methanol and ethyl acetate extracts led to an increase in the expression of TP53 compared to the negative control, while, compared to cells treated with positive control (PC), these extracts treated cells did not show any significant deviation in the activation of TP53 in MCF 7 cells. However, cells treated with chloroform extracts exhibited significant deviation in the expression of TP53 compared to the positive control. (Fig. 13).

The BCL-2 expression studies indicated a notable reduction in BCL-2 expression in cells treated with methanol and ethyl acetate extracts compared to those treated with DMSO treated cells (NC) ($p < 0.0001$) (Fig. 12). The extracts treatment in MCF 7 showed a decrease in BCL-2 expression compared to cisplatin (positive control) however, cells did not exhibit any significant deviation in relation to the positive control. (Fig. 12). In HEK-293 cell lines, the treatment with ethyl acetate and chloroform extract did not show significant deviation from the positive control, while the methanol extract ($p < 0.0171$) demonstrated a significant deviation from the positive control.

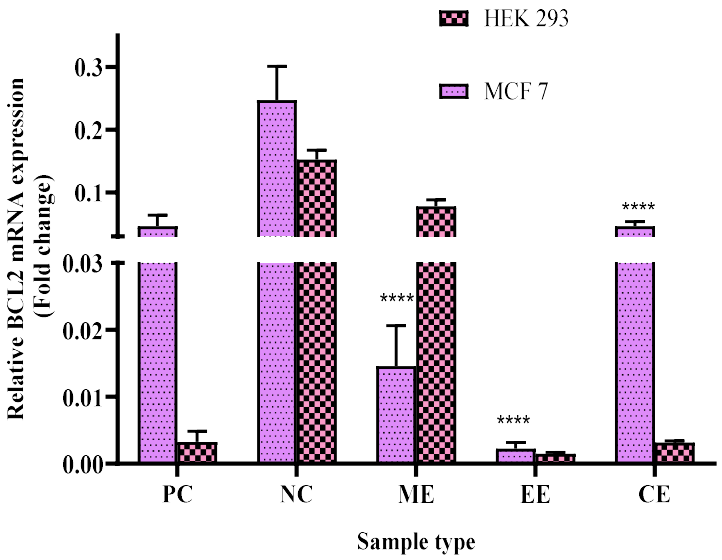


Figure (12): The Changes in Expression of BCL-2 mRNA Induced by the Extracts

The result expressed in Mean ±SD. Where PC, positive control (Cisplatin); NC, negative control (DMSO); EE, ethyl acetate extract; ME, methanol extract and CE, chloroform extract; $^{****}p<0.0001$ (Comparison between the PC and the extracts).

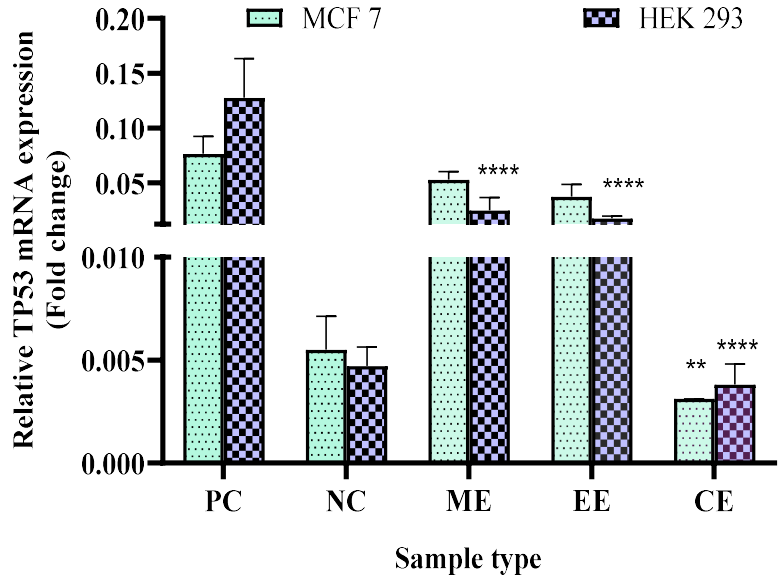


Figure (13): Modulation of TP53 mRNA Expression Induced by the Extracts

The result expressed in Mean ±SD. Where PC, positive control (Cisplatin); NC, negative control (DMSO); EE, ethyl acetate extract; ME, methanol extract and CE, chloroform extract. $^{**}p<0.01$ and $^{****}p<0.0001$ (Comparison between the PC and the extracts).

4.3.5 Immunophenotyping for Caspase Expression Analysis

A significant elevation in caspase expression was detected in the MCF-7 cell line treated with ethyl acetate extract when compared to the positive control (56.6±1.6) (Fig. 14). The ethyl acetate extract demonstrated a significantly higher increase (66.86±2.3, $p<0.0001$) in caspase activity, whereas the chloroform extract (40.7±1.2, $p<0.0001$) and methanol extract (27.9±1.2, $p<0.0001$) demonstrated a significant reduction in caspase 3 expression in comparison to the positive control. On the other hand, all the extract-treated HEK 293 cells showed significant reduction in caspase expression compared to the positive control (Fig. 14).

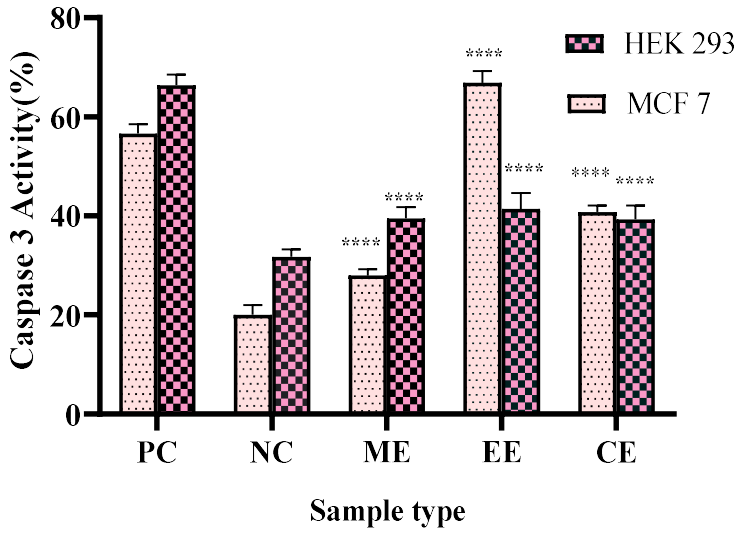


Figure (14): The Percentage of Caspase-3 Activity Induced by the Extracts.

Where PC, positive control (Cisplatin); NC, negative control (DMSO); EE, ethyl acetate extract, respectively, for HEK-293 and MCF-7; ME, methanol extract, respectively, for HEK-293 and MCF-7; CE, chloroform extract, respectively, for HEK-293 and MCF-7.

$^{****}p<0.0001$(Comparison between PC and the extracts).

4.4 Discussion

The immunofluorescent assay revealed significant γH2AX foci formation induced by the methanol and ethyl acetate extracts of *G. colossus*, comparable to the positive control. The accumulated γH2AX foci indicate DNA double-stranded breaks in the nucleus (Mah et al., 2010a; Suleiman et al., 2022b), confirming that these extracts induce DNA damage. Subsequent

cell cycle assays further examined checkpoint activation in breast cancer (MCF-7) and normal (HEK-293) cells.

Cancer cells are characterized by their ability to sustain uncontrolled proliferation, often involving dysregulation of the cell cycle (Patergnani et al., 2020). Most anticancer agents target cell cycle checkpoints and regulatory proteins (Liu et al., 2018). The ethyl acetate extract of *G. colossus* induced G0/G1 arrest in MCF-7 cells, suggesting interference with cyclins, cyclin-dependent kinases, or activation of S-phase checkpoints, preventing entry into the S phase (Liu et al., 2018 ; Thangaraj et al., 2019). Importantly, this arrest was not observed in HEK-293 cells, indicating minimal effects on normal cell lines.

To explore the mechanism of apoptosis, the expression levels of TP53 and BCL-2 were analyzed. TP53 activation in extract-treated MCF-7 cells mirrored the positive control, confirming S-phase checkpoint activation and cell cycle arrest. TP53, a key regulator of cell stress and growth (Marcus et al., 2018b). Downregulated BCL-2 expression, a hallmark of apoptosis (Haldar et al., 1994; Marcus et al., 2018b).

BCL-2 plays a critical role in maintaining mitochondrial membrane integrity, preventing cytochrome C release, and inhibiting caspase activation (Arbab et al., 2012). The observed decrease in BCL-2 expression suggests caspase-mediated apoptotic cell death induced by the extracts, highlighting their potential as anticancer agents. These findings offer promising insights for target-based drug discovery in cancer treatment.

5. Fractional Purification and Molecular Characterization of Bioactive Compounds from *Ganoderma colossus*

5.1 Molecular Identification, GC-MS Analysis and Fractionation of the Sample

5.1.1 Introduction

Accurate identification of a natural source is pivotal when utilized for medicinal purposes (Raja et al., 2017a). Traditionally, external morphological studies are the primary method for identifying fungi. However, for both fundamental and genomic applications, species-level identification of fungi is crucial (Schmitt & Barker, 2009). The *Ganoderma* genus, comprising approximately 80 species with similar morphological characteristics, poses challenges in

identifying various developmental stages. DNA-based identification has emerged as a robust technique for precise identification at the species and genus levels (Raja et al., 2017b).

Natural plant or mushroom extracts may contain numerous compounds, some of which are biologically active or present in trace amounts. Chromatographic techniques play a vital role in purifying natural products and discovering novel drugs with pharmaceutical significance (Bajpai, 2016). Chromatography is a standard method in pharmaceutical applications (Bele et al., 2011). chromatography being a fundamental biophysical method for identifying, separating, and purifying mixture constituents in qualitative and quantitative analyses (Coskun, 2016). Thin Layer Chromatography (TLC) is a simple, rapid, and cost-effective method for identifying components in biological samples (Sasidharan et al., 2011a).

In this study, the mushroom identification was done by using molecular methods, and the partially purification of the extract done through thin-layer chromatography. Cytotoxic analysis was performed on the purified fractions in the human breast cancer cell line, MCF-7. The most potent fraction was further fractionated using column chromatography, with the cytotoxic potential of the column fractions also assessed.

5.1.2 Materials and Methods

5.1.2.1 Molecular Identification

DNA isolation from sequenced samples was conducted using a kit method. The purity of isolated DNA was confirmed through gel electrophoresis. The amplified PCR products were examined using a 1.2% agarose gel. Nucleotide sequences were aligned against the whole nucleotide collection database using the Basic Local Alignment Search Tool (BLAST) search program on the National Centre for Biotechnology Information (NCBI) site. All sequences were deposited into the NCBI database, and accession numbers were acquired along with BLAST results.

5.1.2.2 Thin Layer Chromatography (TLC)

Thin Layer Chromatography was employed (Sasidharan et al., 2011b) to fractionate the crude extract. The methanol and chloroform solvent mixture yielded the most prominent separation of the crude extract.

5.1.2.3 Cytotoxicity Analysis (MTT)

Cytotoxic activity was assessed using the MTT cell viability assay following a 48-hour treatment with fractionated samples of *G. colossus*.

5.1.2.4 Column Chromatography

Positive fractions obtained from TLC were pooled and subjected to silica gel (Merk) column chromatography (Meyers, 2000). The sample was dissolved in a solvent mixture (chloroform: methanol 7:3) and loaded into the silica-packed column, eluted with increasing proportions of chloroform, and methanol.

5.1.2.5 GC-MS (Gas Chromatography Mass Spectrometry) Analysis

GC-MS analysis was conducted at the Sophisticated Analytical Instrument Facility, IIT Bombay, using the AccuTOF-GCv system. This technique is highly effective for screening natural product extracts, providing qualitative and quantitative analysis of organic compounds with exceptional separation power and sensitivity.

The process involved injecting a 2 µL sample of the ethyl acetate extract of *G. colossus* into a DB-5MS capillary column (30.0 m × 0.25 mm, 0.25 µm film thickness). Helium served as the inert carrier gas, and analytes were separated based on differential distribution coefficients within the stationary phase of the column. The retention time of each compound was recorded as the gas phase concentration, determined by its vapor pressure, was controlled.

MS analysis was performed in the 35–550 amu mass range with 70 eV ionization energy and an ion source temperature of 230°C. Volatile compounds were identified by comparing their mass spectra with those in the Wiley (6th edition) and NIST/EPA/NIH (version 1.5a) mass spectra libraries. Results were presented as peak areas corresponding to identify compounds.

5.1.2.6 Statistical Analysis

The methodology mentioned on page no. 10 was followed for statistical analysis.

5.1.3 Results

5.1.3.1 Sample Identification

The ITS rDNA PCR product, a sequence consisting of 569 bases, served as a query in the BLAST search against the GenBank DNA database. The search revealed a high degree of

similarity, with a 98.97% identity match to the *G. colossus* strain. The confirmed sequence has been deposited in the NCBI GenBank database under the accession number ON013931.

5.1.3.2 Thin Layer Chromatography (TLC)

Thin-layer chromatography effectively separated the crude extract into two distinct bands.

5.1.3.3 The Cytotoxicity of Fractions Obtained

Following thin-layer chromatography analysis, two fractions were separated and subsequently pooled. The cytotoxicity analysis of these two bands demonstrated that the second band exhibited a higher potential for cytotoxicity, as indicated by an IC50 value of (25.3±4.2), compared to the first fraction (Table 3).

Table (3): The IC50 Value of Cytotoxicity Assay of TLC Fraction

Mean IC50 Value ± SD	
F1	F2
135.97±1.21	25.31±4.21

F1; Thin layer chromatography fraction 1 and F2; Thin layer chromatography fraction 2

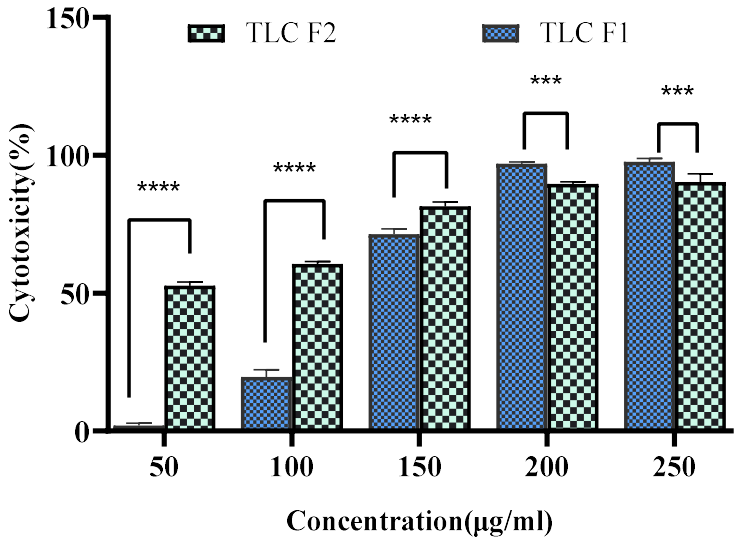


Figure (15): The Cytotoxicity Analysis of the TLC Fractions of *G. colossus*.

Where F1, TLC fraction 1; F2, TLC fraction 2; $^{***}p< 0.001$ and $^{****}p<0.0001$, (Comparison between the fractions).

5.1.3.4 Column Chromatography Analysis

Band 2, obtained from the TLC separation and pooled, underwent further dilution and elution through column chromatography, resulting in the production of 4 fractions. Each fraction, ranging from 7 to 9 ml, was collected, verified using TLC, and utilized in the subsequent study.

5.1.3.5 The Cytotoxicity of the Separated Column Fractions

The fractions obtained from column chromatography were evaluated for their potential cytotoxicity, revealing a dose-dependent increase in cytotoxicity for all present fractions (Fig. 16). Column fraction 3 exhibited the lowest IC50 value for the MTT assay (17.93±2.4), followed by column fraction 2 (19.94±1.3), column fraction 4 (25.98±1.8), and column fraction 1 (30.61±1.8) (Table 4).

Table (4): The IC50 Value for Cytotoxicity Assay of Column Chromatography Fractions

Sample	Mean IC50±SD
CF1	30.61±1.8
CF2	19.94±1.3
CF3	17.93±2.4
CF4	25.98±1.8

CF1, column fraction 1; CF2, column fraction 2; CF3 is column fraction 3; CF4, column fraction 4

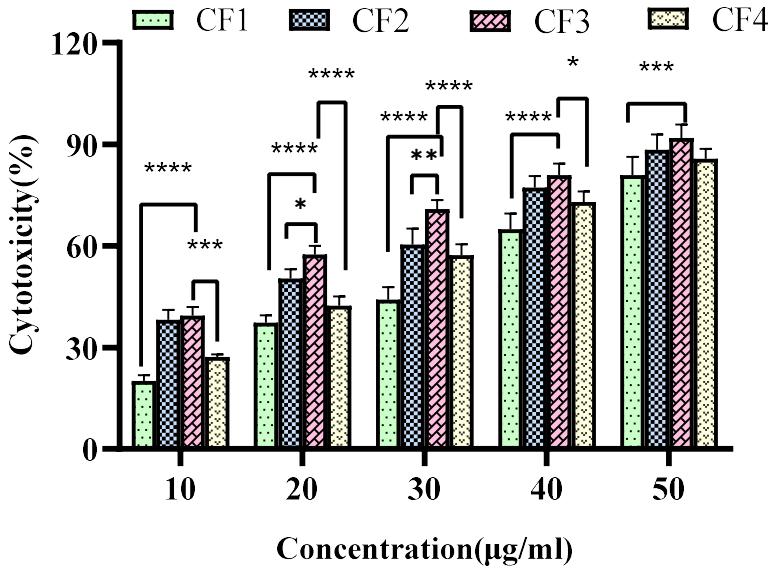


Figure (16): The Cytotoxic Activity of the Column Fractions

CF1; column fraction 1, CF2; column fraction 2, CF3; column fraction 3; *p < 0.05, ** p < 0.01, ***p < 0.001 and ****p < 0.0001, (Comparison between the extracts).

5.1.3.6 GC-MS Analysis

The GC-MS analysis of column fraction 3, identified as the most potent cytotoxic fraction, revealed the presence of long-chain fatty acids, including mono-unsaturated and polyunsaturated fatty acids, along with essential fatty acids (Table 5).

Table 5: GC- MS Analysis of the Column Fraction

Name of the compound	Molecular formula	Nature of compound	Activity	RT	Peak area %
Methyl 14-methylpentadecanate	$C_{17}H_{34}O_2$	ester	Antifungal	26.48	1.1
Methyl Oleate	$C_{19}H_{36}O_2$	ester	Food additive, anti-tummor activity (Dailey et al., 2011)	29.33	9.0
Oleic Acid	$C_{18}H_{34}O_2$	ω9 mono unsaturated Fatty acid	Anti-tumor activity (Kimura, 2002)	31.98	2.6
(9Z,12Z)-octadeca-9,12-dienoic acid/ Linoleic Acid	$C_{18}H_{32}O_2$	Essential fatty acid/ ω -6 poly unsaturated Fatty acid	Decrease blood pressure and serum triglycerides (Raafat, 2018)	48.94	20.3
Linoleoyl Chloride	$C_{18}H_{31}ClO$	Fatty acid	Synthesis of organic polymer (Xue & Chen, 2002)	34.03	19.1
1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	Epoxide	Antitumor, anti-inflammatory(Loof t et al., 2012).	37.16	48.1

Where RT, Retention Time

5.1.4 Discussion

The molecular analysis identified *G. colossus* as the most potent mushroom sample exhibiting cytotoxic activity against cancer cell lines. Initial separation of the potentially active

crude ethyl acetate extract of the mushroom yielded two distinct bands using a methanol and chloroform solvent mixture (7:3). Cytotoxicity assays of these two bands demonstrated that band 2 exhibited a more significant reduction in the number of MCF7 cells compared to band 1 (fraction 1). Subsequently, band 2 (fraction 2) was pooled, collected, and subjected to further fractionation through column chromatography. Among the fractions obtained from column chromatography, fraction 3 demonstrated the highest *in vitro* cytotoxicity (Fig. 16). This suggests the presence of cytotoxic substances in fraction 3, prompting further investigation of this specific fraction.

GC-MS analysis was performed on fraction 3 to identify the chemical compounds present in the sample. The analysis revealed the presence of long-chain and essential fatty acids within the sample. These unsaturated fatty acids have been shown to inhibit necrotic factors associated with tumor presence (Jänicke, 2009; Hofmanová et al., 2014).The study conducted by Salvatore et al. (2020) revealed the presence of oleic acid in *Ganoderma lucidum*, while the research by Gao et al. (2012) demonstrated that a C-19 fatty acid derived from mushrooms could alleviate tumor cell progression and promote apoptosis in HL-60 cells. This further supports the potential anticancer properties of the compounds identified in *G. colossus* fraction 3.

5.2 Validating Anticancer potential of Isolates - Molecular Docking and Apoptotic Assays

5.2.1 Introduction

Apoptosis is a highly organized and coordinated cellular process that occurs in various physiological and pathological conditions. Excessive apoptosis is implicated in degenerative disorders, while insufficient apoptosis is a hallmark of cancer (Ekshyyan & Aw, 2005). Malignant cells often lose their normal properties, such as apoptosis and contact inhibition, contributing to their resistance to death. The intricate mechanism of apoptosis involves multiple pathways (Wong, 2011).

The overexpression of EGFR has been correlated with the loss of estrogen receptor and poor prognosis in breast cancer (Badve et al., 2011). Cancer cells resist most of the current chemotherapy drugs, thus the study focused on EGFR as a potential target for breast cancer treatment. Additionally, HER-2, a vital receptor for breast cancer diagnosis, was included in the docking studies.

BCL-2 is a therapeutic target for some anti-tumor drugs. Inhibitors of BCL-2 induce apoptosis by interacting with a hydrophobic cleft in the BCL-2 molecular structure, thereby

blocking its binding to other pro-apoptotic protein partners like Bax (Radha & Raghavan, 2017). Overexpression of BCL-2 pro-survival proteins or p53 inactivation, leading to aberrant apoptosis pathway activation, is a common cause of most tumors (Adams & Cory, 2017). This study aimed to characterize the expression modulation of two crucial growth regulatory genes, epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER 2), anti-apoptotic protein, BCL-2 and MDM2 a P53 inhibitor by using *in silico* method. Study also included the apoptotic mechanism of the partially purified fraction by *in vitro* methods.

5.2.2 Materials and Methods

5.2.2.1 Computational Studies

AEE788 (PDB ID: 2J6M), TAK-285 (PDB ID: 3RCD) and (PDB ID: 4LXD were taken as the template for docking studies receptor structure.

5.2.2.1.1 Ligand Preparation

The structure of all ligands was downloaded from PubChem database and the geometries of all compounds were prepared using LigPrep module, Schrödinger (Schrödinger, LLC, NewYork, USA). Ionized and tautomeric states of the ligand was generated using Epik program and energy minimization was performed using optimized potential for liquid simulations (OPLS3) force field.

5.2.2.1.2 Protein Preparation and Molecular Docking

The receptor template was prepared using protein preparation wizard of Schrodinger by adding hydrogen atoms to the polar atoms, incorporating protonation states in the residues, optimizing hydrogen bond networks, and minimizing energy using an OPLS-3 force field with a RMSD cut of 0.30 Å. A grid was generated, AEE788 and docking of the prepared ligands into this grid using extra precision (XP) method. The binding energy was predicted using the Prime Molecular Mechanics-Generalized Born Surface Area (MM-GBSA). The strain energies of the ligand conformation in the protein ligand complex were also considered while computing the binding energy of the complex. Prime calculates the binding free energy using the equation:

$$\Delta G(bind) = E_complex\ (minimized) - (E_ligand\ (minimized) + E_receptor\ (minimized)$$

5.2.2.2 Cell Cycle Analysis by Flow Cytometry

MCF-7 cells (0.5 million cells/ml) were treated with various extracts of *G. colossus* for 48 hours and then processed for flow cytometry analysis following the method (Pozarowski & Darzynkiewicz, 2004).

5.2.2.3 Apoptosis Analysis by Annexin Staining

Apoptosis analysis was conducted using the flow cytometry method as outlined by Lakshmanan and Batra (2013) in MCF-7 cells. The analysis was performed using FlowJoX 10.0.7 software.

5.2.2.4 RT-PCR for mRNA Detection

Expression of antiapoptotic and checkpoint genes (BCL-2 and TP53) was analyzed using specific primer sequences. GAPDH served as the housekeeping gene. All samples underwent processing in technical triplicate as well as biological triplicate. The ΔΔCt value was calculated using the Ct values of the housekeeping gene and the no-template controls (NTCs).

5.2.2.5 Statistical Analysis.

The methodology mentioned on page 10 was employed for statistical analysis.

5.2.3 Results

5.2.3.1 Molecular Docking

The findings from the ligand-protein interaction in the in silico docking study are summarized in Table 6. The analysis revealed that the compound (9Z,12Z)-octadeca-9,12-dienoyl chloride exhibited a higher interaction with the antiapoptotic factor BCL-2, showing a binding energy of -82.73 kcal/mol. In contrast, methyl oleate/methyl cis-9-octadecenoate demonstrated a binding energy of -69.82 kcal/mol. The docking score for the crystal ligand control was -114.37 kcal/mol. Octadeca-9,12-dienoyl chloride showed stronger interaction with EGFR, having a binding energy of -69.64 kcal/mol, compared to the control with an optimum binding energy of -66.63 kcal/mol. The compound 1,2-15,16-diepoxyhexadecane exhibited the lowest binding energy of -55.70 kcal/mol. Methyl octadic-9-enoate presented a significant binding interaction with the HER2 receptor, showing a binding energy of -85.16 kcal/mol. Additionally, (9Z,12Z)-octadeca-9,12-dienoic acid/Linoleic Acid had a lower binding energy of -162.26 kcal/mol compared to the control. These findings highlight the potent binding interactions of various compounds with their respective molecular targets, supporting their potential as effective therapeutic agents.

Table (6): Docking Score of Bioactive Compounds with BCL-2(antiapoptotic factor), EGFR and HER2 (MCF-7 cells receptors)

Ligands	PubMedID	Binding Energy (Kcal/mol)		
		Anti-Apoptotic factor BCL2	EGFRkinase domain	HER2 kinase domain
Methyl 14-methylpentadecanoate	21205	-80.02	-58.11	-77.65
(9Z,12Z)-octadeca-9,12-dienoic acid/ Linoleic Acid	5280450	-81.30	-59.56	-56.36
Methyl oleate/Methyl cis-9-octadecenoate	5364509	-69.82	-68.27	-85.16
Oleic acid/ (Z)-octadec-9-enoic acid	445639	-80.93	-59.21	-56.67
Linoleoyl chloride/(9Z,12Z)-octadeca-9,12-dienoyl chloride	9817754	-82.73	-69.64	-76.26
1,2-15,16-Diepoxyhexadecane	543423	-74.09	-55.70	-82.14
Crystal ligand(control)	-	-114. 37	-66.63	- 162.26

5.2.3.2 Cell Cycle Analysis

Cell cycle analysis of the samples, along with the controls, was conducted to identify the checkpoints where the cell cycle was arrested. In comparison to the negative control (8.7%), the MCF-7 cells treated with the sample exhibited a significant arrest ($p < 0.0021$) at the sub G0 stage, with a percentage of 15.59%.

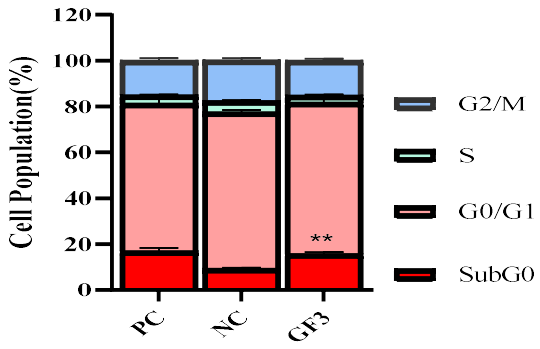


Figure (17): The Percentage Distribution of Cells in Different Stages of Cell Cycle.

PC; positive control, NC; negative control, CF3; Column fraction 3;

**p<0.01 (Comparison between the NC and the column fraction 3).

5.2.3.3 Apoptosis Activity of Purified Fraction 3

Annexin V staining revealed that the sample has the ability to induce apoptosis in MCF-7 cells. The tested sample demonstrated apoptosis induction in treated cells, with comparable findings to cisplatin (16.5 ± 2.4%) (Fig. 18). The number of apoptotic cells in the test sample significantly increased ($p < 0.0001$) to 14.92 ± 3.2% compared to the untreated group (0.54 ± 0.34%). Moreover, there was a substantial reduction (83.7 ± 3.5%; $p < 0.0007$), in the number of live cells when treated with the sample. The standard drug, cisplatin (15 µg/ml), also exhibited a reduction in the number of live cells (79.8 ± 3.07%). The extract treatment did not induce any significant change in the number of live cells, apoptotic and necrotic cell compared to positive control.

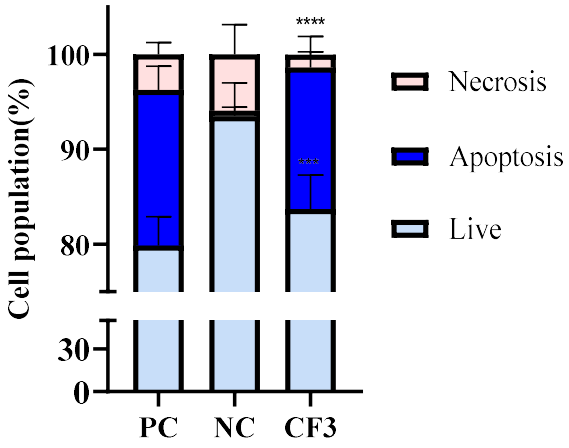


Figure (18): The Percentage Distribution of Live cells, Apoptotic Cells and Necrotic Cells.

PC; positive control, NC; negative control, CF3; Column fraction 3

***p<0.001 and ****p<0.0001 (Comparison between NC and the column fraction 3).

5.2.3.4 TP53 Expression

Expression studies revealed modest yet statistically significant ($p > 0.106$) increases in TP53 expression in cells treated with the sample compared to cells treated with DMSO alone (NC) (Fig. 18). Furthermore, when compared to the positive control, no significant deviation was observed.

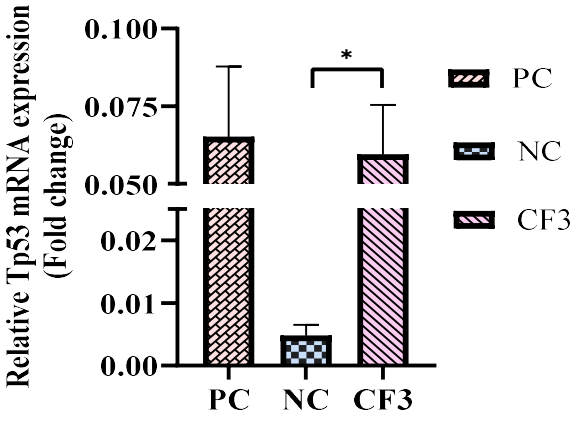


Figure (19): Modulation of TP53 mRNA Expression Induced by the Column Fraction 3
PC; positive control, NC; negative control, CF3; Column fraction 3
*$p<0.05$ (Comparison between the NC and the fraction 3).

5.2.3.5 BCL-2 Expression

The expression analysis revealed a significant down-regulation of BCL-2 in the MCF-7 cell line treated with the sample compared to the NC ($p < 0.0221$) (Fig. 20). In the expression studies, it was observed that the expression of BCL-2 decreased significantly compared to the untreated cells (Fig. 20). This decrease in BCL-2 expression was also evident in cells treated with cisplatin (positive control).

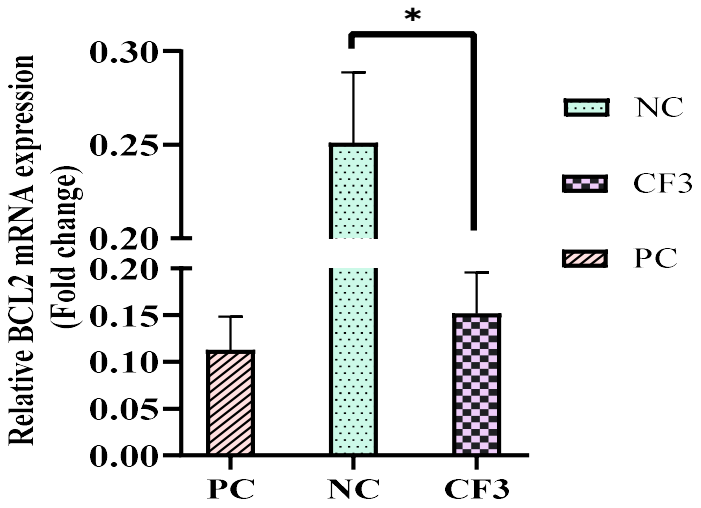


Figure (20): The Changes in Expression of BCL 2 mRNA Induced by the Column Fraction
PC; positive control, NC; negative control, CF3; Column fraction 3;

*p<0.05 (Comparison between the NC and the CF3).

5.2.4 Discussion

Fraction 3 demonstrated significant cytotoxicity compared to other fractions, suggesting its potential role in the observed anti-metastatic and anti-tumor growth effects. Cell cycle arrest, a critical checkpoint where cell duplication and division are completely halted (Li et al., 2019), was evident in fraction 3-treated cells. Cancer cells typically experience cell cycle arrest at various checkpoints (sub G0, G0/G1, S, and G2/M) when exposed to standard cytotoxic drugs (Ryl et al., 2017; Stewart et al., 2003).

GC-MS analysis identified six compounds in fraction 3. In silico docking studies predicted strong interaction between the antiapoptotic factor BCL-2 and (9Z,12Z)-octadeca-9,12-dienoyl chloride, supporting the *in vitro* findings on BCL-2 expression. Docking studies elucidate the molecular confirmation within the enzyme's active site, predicting binding modes, interaction patterns, and functional group proximities involved (Gulipalli et al., 2017).

The ligand interactions with EGFR and HER2 also exhibited significant binding, with compounds from the extract. EGFR inhibitors block critical steps in cell growth and survival, potentially mitigating the adverse effects of cancer treatments (Li et al., 2024) To further explore this mechanism, docking studies targeted MDM2, a negative regulator of p53 (Mah et al., 2010b;

Hernández & El-Deiry, 2021) . The findings revealed that compounds from fraction 3 bind to MDM2 (Figure 5), potentially inhibiting the p53-MDM2 interaction.

Cell cycle analysis showed the sample's ability to arrest the cell cycle at the sub-G0 stage, indicative of apoptosis (Nunez, 2001). The induction of apoptosis is a critical mechanism by which anticancer drugs control tumor cell growth (Niu & Chen, 2010). To understand the apoptotic mechanism, we studied the expression of the proapoptotic factor P53 gene and the anti-apoptotic factor BCL-2. Our findings indicated that the sample down-regulated the tumor suppressor gene BCL-2 and activated the P53 gene.

Tumor suppressors maintain tissue homeostasis by regulating cell survival through DNA damage repair or by inducing apoptosis. Mutations in tumor suppressor genes, resulting in loss of function, contribute to approximately 50% of human cancers by either halting the cell cycle or triggering apoptosis in response to cellular stressors such as hypoxia and DNA damage (Shen & White, 2001; Ozaki & Nakagawara, 2011).

Summary and Conclusion

Natural products have gained attention for their lower toxicity and greater health benefits compared to conventional cancer treatments. Traditional knowledge of cancer treatment has led to the discovery of new anticancer agents from natural sources like mushrooms and plants. These natural products specifically target cancer cells while sparing normal cells. Various medicinal mushrooms, such as those from the genera *Ganoderma* and *Phellinus*, have shown promise in this context.

This study focused on comparing the influence of different solvent polarities on the antioxidant and cytotoxicity profiles of selected mushroom samples, with the goal of identifying the most effective mushroom and solvent extract. The chosen mushroom, *Ganoderma colossus*, was further investigated for its anticancer mechanism after purification through chromatographic methods.

Key Findings:

• The study utilized solvents with varying polarities, revealing differing extraction yields for each solvent. This highlights the importance of solvent choice in achieving maximum extraction efficiency.

• Phytochemical analysis of the mushroom samples identified the presence of phenolics, flavonoids, sugars, alkaloids, tannins, terpenoids, steroids, and proteins.

• Among the mushrooms studied, the methanol extract of *Ganoderma colossus* demonstrated the highest antioxidant activity, while *Phellinus pini* showed significant phenolic and flavonoid content. The selection of solvent significantly affected the observed phenolic content.

• Cytotoxicity assessments across different solvent extracts were conducted on breast cancer cells (MCF-7 and HeLa) and normal cells (HEK-293). The ethyl acetate extract consistently exhibited the highest cytotoxicity, with *Ganoderma colossus* showing the strongest activity against MCF-7 cells while maintaining lower toxicity to normal cells.

• Mechanistic studies of *Ganoderma colossus* revealed its ability to induce cell cycle arrest, DNA damage, and apoptosis in MCF-7 cells. The sample upregulated the tumor suppressor gene p53, down-regulated the antiapoptotic factor BCL-2, and increased expression of caspase-3.

• Fractionation and characterization through GC-MS analysis identified unsaturated and essential fatty acids with potential anticancer properties.

• Molecular docking studies predicted strong interactions of these compounds with MCF-7 cell receptors (EGFR and HER2), the antiapoptotic factor BCL-2 and the p53 inhibitor MDM2.

• *In vitro* investigations confirmed the anticancer activity of the partially purified fraction in the MCF-7 cell line. Further research is necessary to isolate the final pure compound responsible for the observed anticancer effect.

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Presentations in conferences/seminars

- Oral presentation of a paper entitled "Biochemical and Genotoxicity Analysis of Mushroom *Phellinus pini"* in 42nd Annual Conference of Indian Association of Biomedical Scientists, Recent Trends and Advances in Biomedical Sciences – an Integrated Approach, on 25–27th November 2021, NITTE, Mangaluru.

- Oral presentation of a paper entitled "Fractionation and Assessment of Cytotoxicity of Mushroom *Ganoderma colossus*" in Two-day National Conference on Exploring Synergies: Insights in Lifescience and Structural Biology (ESILS-2024) held on 3rd and 4th April 2024 organised by Département of Biotechnology and Microbiology, Kannur University in association with Science and Engineering Research Board(SERB), Département of Science and Technology(DST), Govt of India & Kerala State Council for Science, Technology and Environment(KSCSTE) at Dr. Janaki Ammal Campus, palayad, Kannur University.

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